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(19)


## (54) INSULIN DERIVATIVES


(71) We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, a British Corporation established by Statute, of Kingsgate House, 66—74, Victoria Street, London, S.W.1, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—


This invention relates to insulin derivatives.

As more comprehensive methods for the detection of diabetes mellitus are introduced, and as the normal expectation of life becomes longer, the recorded incidence of this disease is increasing steadily. Present treatment consists of dietary control usually in combination with insulin injections or with an oral antidiabetic drug, and frequently injections once or twice daily are necessary throughout the life of the patient. Even with such treatment the patient's blood sugar level varies considerably from normal necessitating a strict diet. Oral drugs are suitable only in mild cases of diabetes and are now considered to have certain undesirable side effects. In addition to the above mentioned disadvantages of present treatment, a proportion of diabetics produce antibodies to insulin and become increasingly resistant to its action.

It is desirable to produce therapeutic agents which provide better control of blood sugar level than those used in present methods of treatment. To this end, research has been pursued into the properties of insulin derivatives, a field in which in spite of the efforts of many investigators few definite conclusions have emerged hitherto, due largely to failure to separate and adequately identify the individual components of the complex mixture which results from acylation and other reactions to which the parent insulins have been subjected.

Our approach is directed towards the development of a range of insulin derivatives in which the combination of substituent groups is such as to give rise to an improved profile of hypoglycaemic effect. In order to achieve this aim the type of substitution at the primary amino groups of the insulin molecule is of the greatest importance, and in the specification of UK patent number 1,408,757 we describe a group of insulin derivatives including $B_1$(phenylalanine) - N - carbamyl insulin and also insulins in which the primary amino group of the $A_1$(glycine) amino acid unit, and optionally also the primary amino group of one or both of the $B_2$(phenylalanine) and $B_{29}$(lysine) amino acid units, carries a carbamyl group.

It has now been found that carbamyl substitution of the primary amino group of the $B_{29}$ amino acid unit, in the absence of substitution at the $A_1$ and $B_1$ groups, leads to a level of activity in the mouse convulsion assay which is comparable to that resulting from carbamyl substitution at the primary amino group of the $A_1$, $B_1$, $A_1$ and $B_1$ or $A_1$, $B_1$ and $B_{29}$ amino acid units.

Accordingly the present invention comprises an N-substituted insulin in which the primary amino group of the $B_{29}$(lysine) amino acid unit carries a carbamyl group, N-substitution being absent from the primary amino group of the $A_1$(glycine) and $B_1$(phenylalanine) amino acid units.

Although further substitution of the molecule, other than N-substitution at the primary amino group of the $A_1$ and $B_1$ amino acid units, is envisaged as being within the scope of the present invention, the compound $B_{29}$(lysine) - N - carbamyl insulin in which only the $B_{29}$ amino group is substituted is itself of particular interest.

Introduction of a carbamyl group ($NH_2CO$—) in accordance with the invention to effect replacement of hydrogen thereby in the primary amino group of the $B_{29}$ amino acid unit is most conveniently effected by an indirect route which involves blocking of the $A_1$ and $B_1$ amino groups, followed by carbamylation of the $B_{29}$ amino group and subsequent removal of the blocking groups.

Various blocking groups may be employed, for example the trifluoroacetyl group. However, it is preferred to use as a blocking group an acyl group which contains a carboxyl group,

said carboxyl group being linked through an unsaturated carbon-carbon bond to the carbonyl radical of the acyl group. Blocking groups of particular interest are those containing an acyl group derived from maleic acid or from a substituted maleic acid in which one or both of the =CH group hydrogen atoms is replaced by a substituent. The substituent may be, for example, an alkyl group of 1 to 4 carbon atoms, particularly of 1 or 2 carbon atoms, and especially methyl, or a substituted or unsubstituted alkylene group of 3 or 4 carbon atoms, particularly n-propylene and especially n-butylene, which links the two =CH group carbon atoms to form a ring. Specific blocking groups which may be used are maleyl, monomethylmaleyl, dimethylmaleyl, and 3,4,5,6-tetrahydrophthalyl (these terms are used herein to indicate monovalent radicals, for example maleyl is

HOOC . CH=CH . CO—).

The acyl blocking groups described above, including the trifluoroacetyl group, may be introduced by various of the procedures commonly employed for acylation. Thus they may be inserted by reaction of the insulin with the corresponding dicarboxylic acid, for example in the presence of a carbodiimide reagent, or with a suitable functional derivative thereof, for example the acid chloride or anhydride or an activated ester such as those with p-nitrophenol or N-hydroxysuccinimide. Acylation of insulins with dicarboxylic acid functional derivatives, for example anhydrides, is very conveniently amenable to control. If the reaction is carried out with a relatively small excess of acylating agent, preferably of from 2 to 6 moles per mole of insulin, and in an aqueous medium at neutral or mildly alkaline pH, for example 7 to 8, the reaction proceeds in high yield with the formation of the $A_{1,}B_3$-disubstituted derivative. At a pH in the contemplated range the reaction is uncomplicated by O-acylation of tyrosine residues because stable O-acyl derivatives are not formed under these conditions. In most cases the resulting blocked derivatives may conveniently be separated from the reaction mixture by desalting followed by chromatographic separation, further desalting and lyophilization.

Introduction of the carbamyl group on the $B_{29}$ amino group may be effected with various carbamylating agents but alkali metal cyanates are preferred and in particular sodium cyanate or especially potassium cyanate. As the $A_1$ and $B_1$ amino groups are already blocked a large excess of the carbamylating agent may conveniently be used and to ensure a suitable rate of reaction it is preferred that the reaction medium is relatively concentrated in cyanate, for example from 0.5 to 1M.

The trifluoroacetyl blocking group may be removed either at neutral pH if hydroxylamine is employed for the purpose or at an alkaline pH of 10 to 11 if this is not the case. In contrast, the carboxyl group-containing acyl groups described above may be removed by acid hydrolysis, the exact conditions varying somewhat according to the group involved. It will be appreciated that the groups are desirably removable at a pH which is not too acid, thereby avoiding the possibility of reaction at other parts of the molecule, but which also are not too labile at a pH approaching neutrality, thereby avoiding the possibility of premature removal of the group. Of the various groups, the tetrahydrophthalyl and maleyl groups, and especially the monoethylmaleyl group particularly fulfill these conditions. Thus these groups are preferably removed, in an acceptable time span, by the use of a pH in the range from 3 to 4, particularly about 3.5. The monomethylmaleyl group is removable at a pH of 3.5 and a temperature of 37° C in about 18 hours whilst the maleyl and tetrahydrophthayl groups require from 90 to 180 hours under the same conditions. The dimethylmaleyl group and similar groups are less suitable, firstly since they show some degree of lability even at a pH of 7 and secondly as they require treatment at an acid pH, for example 3.5, then an alkali pH, for example 9.5, then again at an acid pH, for example 3.5, to effect full removal of the group (this is believed to be due to the formation of an intermediate of a different type to that obtained with the other acyl groups). Preferably, the medium for effecting the deacylations contains guanidine hydrochloride, since this solubilises the insulin derivative which would otherwise generally not be sufficiently soluble at a pH of about 3.5 due to the influence of two acyl substituents. Sufficient guanidine hydrochloride is used to effect solution, a concentration of about 5M in the reaction medium being preferred.

The present invention is applicable to various forms of insulin and particularly the porcine and bovine insulins which have been used clinically for many years in the treatment of diabetes and other disorders. It is also applicable to synthetic insulins of this type and to synthetic human insulin. As with the parent insulins, zinc may be present in some form in N-substituted insulins according to the present invention. Such insulins may be formulated as pharmaceutical preparations in the same way as the parent insulins and may be used clinically at lower, comparable, or higher dosage levels. Thus the normal daily dosage of insulin is from 20 to 80 international units per day for adults, and for resistant patients more than 200 units and in some cases over 500 units of standard insulin. The derivatives of this invention can be prepared as solutions,

suspensions, or freeze-dried preparations. A typical solution formulation is of a neutral or physiological pH and contains sodium acetate 0.136% w/v, sodium chloride 0.7% w/v and methyl hydroxybenzoate 0.1% w/v in pyrogen-free water.

Although it is most often advantageous to prepare the $B_{29}$—N—carbamylated insulin in substantially pure form, i.e. substantially free from by-products of manufacture, it is nevertheless possible to administer the compound as one component, for example the major one by weight of a mixture of insulin derivatives. Such a mixture may be obtained from the carbamylation reaction or more preferably by admixture of a physiologically acceptable substantially pure $B_{29}$-N-carbamylated insulin with other insulin derivatives, or with insulin itself. The use of such mixtures can lead to optimisation of effects or other advantageous control of therapy.

Accordingly the present invention further includes a pharmaceutical preparation which is preferably one for parenteral administration, comprising as an active ingredient an N-substituted insulin as defined above, together with a physiologically acceptable diluent or carrier.

The invention is illustrated by the following Examples:

EXAMPLE 1:

Preparation of $B_{29}$(lysine)-N-carbamylinsulin.

(a) $A_{21}B_1$-$N_\alpha N^1$-di(methylmaleyl)insulin.

A solution of zinc-free insulin (115 mg, 20μ mole) in 0.2M phosphate buffer of pH 7.0 (6 ml) is treated with a solution of methyl maleic anhydride (4.5 mg, 40μ mole) in anhydrous dioxan (0.4 ml) and the reaction mixture is stirred at room temperature for 2 hours. The resulting mixture of N-methylmaleyl insulins is desalted by gel filtration on a column (35 cm × 2.5 cm) of Sephadex G—25 (coarse grade) in 0.05% N-ethylmorpholine at pH 9.0 and isolated by lyophilization. The freeze-dried mixture is dissolved in 5 ml of 0.06M tris chloride [tris is an abbreviation for tris(N-hydroxymethyl)amino methane] of pH 7.5 containing 8M urea (freed from cyanate by acidification) and added to a DEAE (Diethylaminoethyl) Sephadex (registered Trade Mark) A—25 column (80 cm × 1.5 cm) which is equilibrated and developed initially with 200 ml of the same buffer. A linear gradient of tris chloride is then applied to the column up to a limiting concentration of 0.25M by passing 0.25M tris chloride into a mixing vessel containing 250 ml of the starting buffer. The flow rate used is 15 ml per hour and the elution of material is monitored via the UV absorption of the eluate. Using this chromatographic procedure, the $A_{21}B_1$-$N_\alpha N^1$-di(methylmaleyl) insulin is eluted from the column after the unreacted insulin, the N-monomethylmaleyl insulins and $A_{21}B_{29}$-$N_\alpha N^1$-di(methylmaleyl) insulin. Desalting in a similar manner to that described above, followed by lyophilization gives a yield of 55 mg, the presence of the free ε—$NH_2$ being confirmed by trypsinisation and the absence of free α—$NH_2$ by lack of carbamylation.

(b) $A_{21}B_1$ - $N_\alpha N^1$ - di(methylmaleyl) - $B_{29}$-$N^{\varepsilon\prime}$-carbamylinsulin.

A solution of $A_{21}B_1$-$N_\alpha N^1$-di(methylmaleyl) insulin (11 mg) in 3 ml of 0.5M tris HCl buffer chloride at pH 8.5 is mixed with 3 ml of aqueous 1M potassium cyanate and the reaction mixture is allowed to stand at room temperature for 16 hours. Monitoring of the degree of reaction by trypsinisation of an aliquot of the reaction mixture (for detection of free ε—$NH_2$) typically shows 90% carbamylation at this stage. The reaction mixture is desalted by addition to a column of Sephadex G—25 operated with 0.2% v/v N-ethylmorpholine as eluant and the protein is isolated by lyophilization of the eluant.

(c) $B_{29}$-N-carbamylinsulin.

The freeze-dried residue from (b) above, containing $A_{21}B_1$ - $N_\alpha N^1$ - di(methylmaleyl)-$B_{29}$-$N^{\varepsilon\prime}$-carbamylinsulin, is taken up in 3 ml of 1M sodium citrate of pH 3.5 which is 5M in guanidine hydrochloride, and incubated at 37° C for 14 hours. The mixture is neutralised by the addition of 1M tris HCl chloride of pH 9.5 and then desalted using a column of Sephadex G—25 with 0.2% v/v N-ethylmorpholine as eluant. The protein is isolated from the eluant by lyophilization and the freeze-dried material is taken up in 2 ml of 0.08M tris HCl chloride of pH 7.1 which is 8M in urea (freed from cyanate by acidification) and added to a DEAE Sephadex A—25 column (40 cm × 1.5 cm) which is equilibrated and developed initially with the same buffer and then with a linear gradient of NaCl from 0 to 0.2M. The elution of the column is followed by measurement of E277 in the UV range, the eluate being collected in a series of tubes as fractions of volume 2 ml. The $B_{29}$-N-carbamylinsulin typically emerges at about tube 25 and is the major peak of UV absorbing material from the column. The fractions containing this compound are combined, desalted using a column of Sephadex G—25 with 0.2% v/v N-ethyl morpholine as eluant and lyophilized to give $B_{29}$-N-carbamyl insulin in 6.5 mg yield, the compound being characterised by trypsinisation (no detectable release of alanine and hence no free ε—$NH_2$) and carbamylation (two free ε—$NH_2$). The compound moves as a single band on polyacrylamide electrophoresis at pH 8.5 in 8M urea.

EXAMPLE 2:

Preparation of $B_{29}$(lysine)-N-carbamylinsulin.

In variations of the procedure used in Ex-

ample 1, maleyl, tetrahydrophthalyl or dimethylmaleyl blocking groups are used in place of methylmaleyl blocking groups.

The $A_{21}B_1$-$N_\alpha N'$-disubstituted insulin is in each case prepared by a similar procedure to that described in Example 1(a) using the same molar proportion of the appropriate anhydride, with the exception that in the case of the dimethylmaleyl blocking group the reaction is effected at a higher pH of about 9.0 to prevent labilization and the derivative is isolated by an alternative technique consisting of desalting at pH 10 on a $CO_3^=/HCO_3^-$ column followed by lyophilization.

The $A_{21}B_1$ - $N_\alpha N^1$ - disubstituted - $B_{29}$-$N^{\varepsilon 1}$-carbamylinsulin is in each case prepared by a similar procedure to that described in Example 1(b) with similar variations in the case of the dimethylmaleyl group to those described above.

Removal of the blocking groups requires some modification in each case of the conditions given in Example 1(c), the maleyl and tetrahydrophthalyl groups requiring treatment at 37° C in 1M sodium citrate of pH 3.5 which is 5M in guanidine hydrochloride for approximately ten times as long as given in that Example, and the dimethylmaleyl group requiring treatment at pH 3.5, then at 9.5, then again at pH 3.5. The working up procedure to provide $B_{29}$-N-carbamylinsulin is in each case similar to that described in Example 1(c).

WHAT WE CLAIM IS:—

1. An N-substituted insulin in which the primary amino group of the $B_{29}$(lysine) amino acid unit carries a carbamyl group, N-substitution being absent from the primary amino group of the $A_1$(glycine) and $B_1$(phenylalanine) amino acid units.

2. $B_{29}$(lysine)-N-Carbamylinsulin.

3. Physiologically acceptable substantially pure $B_{29}$(lysine)-N-carbamylinsulin.

4. A process for the preparation of an N-substituted insulin according to claim 1, which comprises reacting an insulin in which the primary amino group of each of the $A_1$ and $B_1$ amino acid units is substituted by a blocking group with a carbamylating agent to effect carbamylation of the primary amino group of the $B_{29}$ amino acid unit, and thereafter removing said blocking groups on the $A_1$ and $B_1$ amino acid units.

5. A process for the preparation of an N-substituted insulin according to claim 1, which comprises removing the $A_1$ and $B_1$ amino acid blocking groups from an insulin in which the primary amino group of each of the $A_1$ and $B_1$ amino acid units is substituted by a blocking group and the primary amino group of the $B_{29}$ amino acid unit carries a carbamyl group.

6. A process according to Claim 4 or 5, in which the N-substituted insulin obtained by the process is $B_{29}$(lysine)-N-carbamylinsulin.

7. A process according to Claim 4, 5 or 6 in which the blocking group is trifluoroacetyl.

8. A process according to Claim 4, 5 or 6 in which the blocking group is an acyl group which contains a carboxyl group, said carboxyl group being linked through an unsaturated carbon-carbon bond to the carbonyl radical of the acyl group.

9. A process according to Claim 8, in which the blocking group is a mono-valent acyl radical derived from a dicarboxylic acid selected from maleic acid and mono- and di-substituted maleic acids.

10. A process according to Claim 9, in which the blocking group is a mono-basic acyl residue of a dicarboxylic acid selected from maleic acid and substituted maleic acids in which one or both of the =CH groups is substituted by an alkyl group of 1 to 4 carbon atoms or in which both =CH groups are substituted by an alkylene group of 3 or 4 carbon atoms which links them to form a ring.

11. A process according to Claim 10, in which the blocking group is maleyl, monomethylmaleyl, dimethylmalyl or 3,4,5,6-tetrahydrophthalyl.

12. A process according to Claim 11, in which the blocking group is monomethylmaleyl.

13. A process according to Claim 4 or any of Claims 6 to 12 as dependent on Claim 4, in which carbamylation is effected with an alkali metal cyanate.

14. A process according to Claim 13, in which carbamylation is effected with potassium cyanate.

15. A process according to Claim 7 or Claim 13 or 14 as dependent on Claim 7, in which the trifluoroacetyl blocking group is removed by a treatment comprising the use of hydroxylamine at neutral pH or of hydrolysis at a pH of 10 to 11.

16. A process according to any of Claims 8 to 12 or Claims 13 to 14 as dependent on any of Claims 8 to 12, in which the acyl blocking group is removed by a treatment comprising the use of hydrolysis at acid pH.

17. A process for the preparation of an N-substituted insulin according to Claim 4, substantially as described in Example 1 or 2.

18. A process for the preparation of an N-substituted insulin according to Claim 5, substantially as described in Example 1 or 2.

19. An N-substituted insulin whenever prepared according to the process of any of Claims 4 to 18.

20. A pharmaceutical preparation which comprises as an active ingredient thereof an N-substituted insulin according to Claim 1, together with a physiologically acceptable diluent or carrier.

21. A pharmaceutical preparation according to Claim 20 in a form for parenteral administration.

22. A pharmaceutical preparation according to Claim 20 comprising an N-substituted insulin according to any of Claims 2, 3 and 19 as an active ingredient thereof.

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